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Inter-relationships between light and respiration in the control of ascorbic acid synthesis and accumulation in *Arabidopsis thaliana* leaves

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Abstract

The effects of growth irradiance and respiration on ascorbic acid (AA) synthesis and accumulation were studied in the leaves of wild-type and transformed *Arabidopsis thaliana* with modified amounts of the mitochondrial alternative oxidase (AOX) protein. Plants were grown under low (LL; 50 μmol photons m^{-2} s^{-1}), intermediate (IL; 100 μmol photons m^{-2} s^{-1}), or high (HL; 250 μmol photons m^{-2} s^{-1}) light. Increasing growth irradiance progressively elevated leaf AA content and hence the values of dark-induced disappearance of leaf AA, which were 11, 55, and 89 nmol AA lost g^{-1} fresh weight h^{-1}, from LL-, IL-, and HL-grown leaves, respectively. When HL leaves were supplied with L-galactone-1,4-lactone (L-GalL; the precursor of AA), they accumulated twice as much AA and had double the maximal L-galactone-1,4-lactone dehydrogenase (L-GalLDH) activities of LL leaves. Growth under HL enhanced dehydroascorbate reductase and monodehydroascorbate reductase activities. Leaf respiration rates were highest in the HL leaves, which also had higher amounts of cytochrome *c* and cytochrome *c* oxidase (CCO) activities, as well as enhanced capacity of the AOX and CCO electron transport pathways. Leaves of the AOX-overexpressing lines accumulated more AA than wild-type or antisense leaves, particularly at HL. Intact mitochondria from AOX-overexpressing lines had higher AA synthesis capacities than those from the wild-type or antisense lines even though they had similar L-GalLDH activities. AOX antisense lines had more cytochrome *c* protein than wild-type or AOX-overexpressing lines. It is concluded that regardless of limitations on L-GalL synthesis by regulation of early steps in the AA synthesis pathway, the regulation of L-GalLDH activity via the interaction of light and respiratory controls is a crucial determinant of the overall ability of leaves to produce and accumulate AA.

Key words: Ascorbic acid, alternative oxidase, cytochrome *c*, light acclimation, mitochondria, respiration.

Introduction

Plant tissues generally contain abundant amounts of ascorbic acid (AA). However, increases in the contents and stability of this vitamin in fruit and vegetables remain

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Abbreviations: AA, ascorbic acid AOX, alternative oxidase APX, ascorbate peroxidase CCO, cytochrome *c* oxidase DHA, dehydroascorbate DHAR, dehydroascorbate reductase L-GalL, L-galactone-1,4-lactone L-GalLDH, L-galactone-1,4-lactone dehydrogenase HL, high light HPLC, high-performance liquid chromatography IL, intermediate light LL, low light MDHA, monodehydroascorbate MDHAR, monodehydroascorbate reductase ROS, reactive oxygen species.

important considerations in the improvement of food quality and nutrition (Davey *et al.*, 2000). Current knowledge and understanding of the factors that govern the extent of AA accumulation in plants are far from comprehensive (Bartoli *et al.*, 2005). Developmental triggers and environmental signals, particularly light, influence leaf AA accumulation (Imai *et al.*, 1999; Gatzek *et al.*, 2002). Growth at low light has been found to decrease the abundance of transcripts encoding enzymes involved in AA synthesis such as L-galactono-1,4-lactone dehydrogenase (L-GalLDH) and GDP-mannose pyrosphorylase in some studies (e.g. Tabata *et al.*, 2002) but not others (Pignocchi *et al.*, 2003). Moreover, no relationship between the amount of L-GalLDH protein and the extent of AA accumulation could be found in the leaves of a range of different species, indicating that the abundance of this protein could not be used as a marker/indicator of the capacity for AA accumulation (Bartoli *et al.*, 2005).

The concept that AA has a crucial role in antioxidant defence preventing the accumulation of reactive oxygen species (ROS) as well as in stress protection is well established (Anderson *et al.*, 1983*a*, *b*; Smirnoff, 1996; Tambussi *et al.*, 2000; Müller-Moulé *et al.*, 2002; Bartoli *et al.*, 2004). However, AA is a multifunctional metabolite in plants (Noctor and Foyer, 1998) influencing nearly every aspect of plant biology from mitosis and cell expansion (De Gara *et al.*, 1999; Tabata *et al.*, 2001) to senescence and defence against pathogens (Garg and Kapoor, 1972; Borraccino *et al.*, 1994; Barth *et al.*, 2004; Pavet *et al.*, 2005). The ascorbate/glutathione cycle of hydrogen peroxide detoxification, which was first characterized in plants (Foyer and Halliwell, 1976; Foyer, 1997) and later in animals, comprises ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase. APX, which catalyses the hydrogen peroxide-dependent oxidation of AA to MDHA and subsequently DHA, is an abundant enzyme with a number of isoforms found in different compartments of the cell. MDHA can be re-reduced to AA by MDHAR or by spontaneous dismutation to DHA and AA (Buettner and Jurkiewicz, 1996). DHA is enzymatically reduced by the reaction catalysed by DHAR using glutathione as electron donor, although other as yet uncharacterized glutathione-independent mechanisms of ascorbate regeneration from DHA are clearly present in plant cells (Potters *et al.*, 2004).

Plants make AA via *de novo* synthesis pathways (Wheeler *et al.*, 1998) and carbon skeleton re-cycling networks (Smirnoff *et al.*, 2004). *De novo* synthesis from GDP-mannose can involve two routes, one via L-galactose and the second via L-gulose. In green leaves, L-galactone-1,4-lactone (L-GalL) is derived largely from the L-galactose pathway (Wheeler *et al.*, 1998; Smirnoff *et al.*, 2001, 2004). Alternatively, L-GalL can be formed through the galacturonic acid pathway (Agius *et al.*, 2003). Both the L-galactose and galacturonic acid pathways share a common last step in the oxidation of the AA precursor; L-GalL (Smirnoff *et al.*, 2001). It is generally accepted that the synthesis of L-GalL is rate-limiting for AA synthesis. However, the relationship between AA synthesis and respiration mediated by the last enzyme of the pathway is intriguing in terms of regulation. To date, the importance and relevance of mitochondrial limitations on AA accumulation *in planta* have not been established. The oxidation of L-GalL to AA is accomplished by the reduction of cytochrome *c*, in a reaction catalysed by the inner mitochondrial membrane enzyme L-GalLDH (Siendones *et al.*, 1999; Bartoli *et al.*, 2000). The addition of KCN to intact isolated mitochondria fully inhibits AA formation, demonstrating that oxidized cytochrome *c* is essential for AA synthesis (Bartoli *et al.*, 2000). Furthermore, the activity of L-GalLDH depends on the engagement of mitochondrial complex I. The addition of rotenone, a specific inhibitor of complex I, in the presence of pyruvate and malate blocks the synthesis of AA in mitochondria isolated from *Arabidopsis* leaves (Millar *et al.*, 2003).

A key difference between plant and animal mitochondria in addition to the ability of plant mitochondria to produce AA is the presence of alternative respiratory pathways, which are considered to play a role in the control of ROS formation. In addition to non-proton-pumping NAD(P)H dehydrogenases that by-pass complex I (Rasmusson *et al.*, 1998), plant mitochondria have an alternative oxidase (AOX) that accepts electrons directly from the ubiquinone pool without intervention of the cytochrome *c* oxidase (CCO) pathway through complexes III and IV. These alternative pathways allow uncoupling of electron transfer from ATP production, thus preventing over-reduction of the respiratory electron transport chain that could otherwise occur in situations of major flux restrictions (Day and Wiskich, 1995; Vanlerberghe and McIntosh, 1997; Wagner and Moore, 1997). This regulation diminishes the risk of ROS generation. Accordingly, AOX has been shown to be induced by H_2O_2 (Wagner, 1995), and inhibition or underexpression of the AOX stimulates H_2O_2 production (Popov *et al.*, 1997; Maxwell *et al.*, 1999; Umbach *et al.*, 2005).

Evidence that light and respiration influence the capacity for AA synthesis/accumulation is presented here. It is shown that higher growth irradiance increases the capacity of leaves to synthesize, regenerate, and accumulate AA in the light, and to deplete the AA pool in the dark. Moreover, evidence is presented that the relative capacities of the AOX and CCO pathways, as well as the overall capacity for leaf respiration, also influence the extent of leaf AA accumulation. Hence, AA synthesis is influenced by AOX activity, which also regulates the high energy state of the membrane, draining electrons and preventing ROS production. The close interaction of AA synthesis and AOX activity provides evidence for a concerted interaction at

the level of the mitochondrial electron transport chain, to protect the cell against uncontrolled oxidation (Foyer and Noctor, 2005).

Materials and methods

Wild-type *Arabidopsis thaliana* plants were cultivated for 2 weeks in a growth chamber at 22±1 °C, at an irradiance of 50 μmol photons m^{-2} s^{-1}, with a 10 h photoperiod. Plants were then transferred to either low (LL; 50 μmol photons m^{-2} s^{-1}), intermediate (IL; 100 μmol photons m^{-2} s^{-1}), or high (HL; 250 μmol photons m^{-2} s^{-1}) light for a further 2 weeks, at which point leaves were harvested for the analyses described below.

Production of AOX1a transgenic plants

The AOX1a coding region was amplified from a cDNA λ-Zap library (Lin and Thomashow, 1992) using the following primers containing unique restriction sites: antisense primer #1, 5′-TCTAGACCGATTTGAAACAATGATGATAAC-3′; antisense primer #2, 5′-AAGCTTTTTAATCGAATCAATGATACCCAAT-3′; sense primer #1, 5′-AAGCTTCCGATTTGAAACAATGATGATAAC-3′; and sense primer #2, 5′-TCTAGATTTAATCGAATCAATGATACCCAAT-3′. The AOX1a fragments were cloned into pGEM-T Easy vector and sequenced then subcloned into pKYLX71::35S[2] (Schardl *et al.*, 1987) at *Hin*dIII and *Xba*I sites, behind the duplicate cauliflower mosaic virus (CaMV) 35S promoter, in sense and antisense configurations, respectively. The resulting plasmids were transformed into *Agrobacterium* strain LBA4404 (supplied by Gibco-BRL) by electroporation.

Arabidopsis accession Col-0 *gl1* was grown in soil, at 20 °C with an irradiance of 100 μmol photons m^{-2} s^{-1} and a 12 h photoperiod. Batches of plants were transformed using the floral dip procedure (Clough and Bent, 1998). Sterilized T_1 seeds were sown on plates in MS medium with 1% sucrose, 0.8% phytagar, 30 mg l^{-1} kanamycin, and 300 mg l^{-1} Timentin. Dark green seedlings were transferred to hard selection plates that were identical to the above plates except that they also contained 1.5% phytagar. The seedlings were transferred from hard selection plates to soil in 2 weeks. Thirty-one sense lines and 24 antisense lines were recovered after kanamycin selection. Homozygous, single insertion lines were identified and used for subsequent analysis.

AOX protein determinations in the mitochondria of wild-type and AOX1a transgenic plants

Leaf AOX accumulation was studied using western blot analysis in homozygous lines identified on the basis of kanamycin resistance. AOX proteins were detected either in total leaf extracts or in isolated mitochondria. For whole leaf extracts, samples (0.1 g) were harvested from 3-week-old plants. They were ground in sample loading buffer (800 μl per sample) and centrifuged at 13 000 rpm. for 1 min in a table-top centrifuge. The extracts were then heated in a boiling water bath for 2 min. Samples (10 μl) were then loaded on SDS gels. Alternatively, isolated intact mitochondria were prepared from the leaves of 5-week-old plants that had been treated with 10 mM antimycin to induce the expression of the AOX protein. In these experiments, samples (0.5 g) were harvested from 4-week-old plants. They were placed in 10% MS medium containing 10 μM antimycin A. A gentle vacuum was applied for 5 min to facilitate the penetration of the inhibitor into the leaves. The leaves were then incubated for a further 16 h in the medium at 21 °C in the dark. Mitochondria were then isolated as below and samples of (10 μg) mitochondrial protein were loaded on the SDS gels. Proteins were separated by SDS–PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody to AOX as described (Elthon *et al.*, 1989).

Isolation of intact mitochondria

Mitochondria were isolated as described by Purvis (1997) except that leaves were homogenized in 75 mM MOPS buffer (pH 7.5) containing 600 mM sucrose, 4 mM EDTA, 0.2% (w/v) polyvinylpyrrolidone (PVP)-40, 8 mM cysteine, and 0.2% (w/v) bovine serum albumin (BSA). Homogenates were centrifuged at 3000 *g* for 10 min and the supernatants were centrifuged again at 16 000 *g* for 10 min. The pellet was resuspended in 10 mM MOPS buffer (pH 7.2) containing 300 mM sucrose. Samples were layered onto Percoll gradients consisting of 2.5 ml of 45% Percoll and 7.5 ml of 20% Percoll. The gradients were centrifuged at 26 000 *g* for 15 min. Mitochondria, recovered at the interface between the 20% and 45% Percoll layers, were washed twice with buffer and assayed immediately.

Determination of reduced and total AA content

Reduced AA was measured by high-peformance liquid chromatography (HPLC) as described by Iwase (1992). Leaves were ground in 6% trifluoracetic acid and centrifuged at 13 000 *g* for 5 min. Supernatants were passed through a C-18 column (Bond Elute, Varian) for a partial purification of the sample. The samples were then filtered and injected onto an HPLC system (Shimatzu LC-10Atvp solvent delivery module) equipped with a C-18 column (Varian Chromsep 100×4.6 mm) and detected at 265 nm (Shimatzu UV-Vis SPD-10Avp detector). AA was separated isocratically using 100 mM phosphate buffer pH 3.0 as a running solution at a flux of 0.6 ml min^{-1}. Total ascorbate was measured after reducing DHA by mixing 1 vol. of the sample obtained after the C-18 column and 1 vol. of 100 mM phosphate buffer pH 7 in the presence of 5 mM dithiothreitol (DTT). The reaction was incubated for 10 min and the AA was measured. DHA was calculated as the difference between total and reduced AA.

Measurements of L-GalLDH activity and content

The effect of irradiance on the content and activity of L-GalLDH (EC 1.3.2.3) was measured in membrane protein fractions extracted as previously described (Bartoli *et al.*, 2000). L-GalLDH content in AOX transformants was measured in the same leaf homogenates obtained for the determination of cytochrome *c* as detailed below. The activity of L-GalLDH was measured spectrophotometrically following the increase in the absorbance at 550 nm after addition of 2 mM L-GalL (Bartoli *et al.*, 2000). The relative amounts of the L-GalLDH protein were assayed by SDS–PAGE and western blotting (Tambussi *et al.*, 2000). Blots were probed with anti-maize L-GalLDH antiserum [1:5000 in phosphate-buffered saline–Tween (PBST)] and goat anti-rabbit IgG–horseradish peroxidase conjugate (1:50 000 in PBST), prior to visualization using a chemiluminescence detection kit (Renaissance™, DuPont, Boston, MA, USA) and Kodak X-OMAT XAR5 films according to the manufacturers' instructions. Relative protein content (in arbitrary units) was determined by densitometry of the films.

Assaying the capacity of Arabidopsis *leaves to accumulate AA*

Arabidopsis leaves were detached and incubated in 30 mM L-GalL or water overnight in the dark. At the beginning of the photoperiod, the samples were washed in distilled water and exposed to LL, IL, or HL. Total AA was determined after 6 h of incubation. The capacity for AA production was estimated as the difference between water- and L-GalL-fed leaves. Similarly, AA production was measured in detached leaves of AOX transformants incubated with 30 mM L-GalL in the presence of 0.05% Tween-20 at high irradiance for 4 h. The capacity for AA production was estimated via the difference between leaves incubated with L-GalL and 0.05% Tween-20/water controls.

Respiration measurements

Respiration was measured in isolated mitochondria using a Clark-type oxygen electrode (Hansatech, UK) in a reaction medium (1 ml) consisting of 20 mM MOPS (pH 7.4), 300 mM sucrose, 5 mM $MgCl_2$, 5 mM K_2HPO_4, 10 mM KCl, and 0.1 mM ADP. Respiration by detached leaves was monitored using the LD2/3 leaf disc electrode (Hansatech, UK). To assess the maximum capacity of the AOX pathway, the cytochrome *c* pathway was inhibited with 1 mM KCN. The AOX pathway was inhibited with 10 mM salicylhydroxamic acid (SHAM). Leaves were immersed for 3 h prior to assay, in the buffer containing these respiratory inhibitors together with a surfactant agent (0.05% Tween-20) to allow penetration, under low light (10–20 μmol m^{-2} s^{-1}). The leaves were then dried, placed in the oxygen electrode chamber and respiration was measured.

Cytochrome c *detection and CCO activity determinations*

Whole leaves were homogenized in 62.5 mM TRIS–HCl (pH 6.8) buffer containing 2% SDS, 10% (v/v) glycerol, 5% (v/v) mercaptoethanol, 3 M urea, and protease inhibitors [250 μM phenylmethylsulphonyl fluoride (PMSF) and 2 μM leupeptin]. Samples were centrifuged at 13 000 *g* at 4 °C for 10 min and stored at –70 °C. Samples were separated by denaturing gel (15% polyacrylamide) electrophoresis and transferred to nitrocellulose membranes. Cytochrome *c* protein contents were then estimated using monoclonal antibodies (catalogue no. 554002, BD Pharmigen). CCO activity was determined in the same leaf homogenates as those used for L-GalLDH activity. The reaction mixture and method were as described in Bartoli *et al.* (2000).

Assays for MDHAR and DHAR activity

Leaves were ground in a medium containing 0.1 M bicine (pH 7.5), 1 mM EDTA, 10% (v/v) glycerol, 4 mM cysteine, and protease inhibitors (250 μM PMSF and 2 μM leupeptin). The homogenates were centrifuged at 13 000 *g* for 10 min and the supernatants used for the enzymatic determinations. The activities of MDHAR (EC 1.6.5.4) and DHAR (EC 1.8.5.1) were measured essentially as described by De Gara *et al.* (2000). For DHAR measurements, the reaction mixture consisted of 50 mM phosphate buffer (pH 6.5), 0.2 mM DHA, 2.5 mM reduced glutathione, and 50–100 μg of leaf protein.

Results

The effect of irradiance on leaf AA content

Arabidopsis plants grown under different light regimes showed a phenotypic acclimation to light availability (Fig. 1A). The rosette leaves of plants grown under either LL (50 μmol photons $m^{-2}s^{-1}$), IL (100 μmol photons $m^{-2}s^{-1}$), or HL (250 μmol photons m^{-2} s^{-1}) also had different amounts of AA (Fig. 1B). The levels of AA were lower in darkness than in the light, the dark/light variation being most pronounced in the HL plants. In plants grown under LL conditions, there was a dark-dependent decrease in leaf AA content during the night. A dark-induced decline of AA content was observed in the IL-grown plants and it was pronounced in the HL plants, where leaf AA decreased by about half in darkness (Fig. 1B). The dark-induced disappearance of leaf AA occurred at rates of ~11, 55, and 89 nmol AA lost g^{-1} fresh weight (FW) h^{-1}, for LL-, IL-, and HL-grown leaves, respectively.

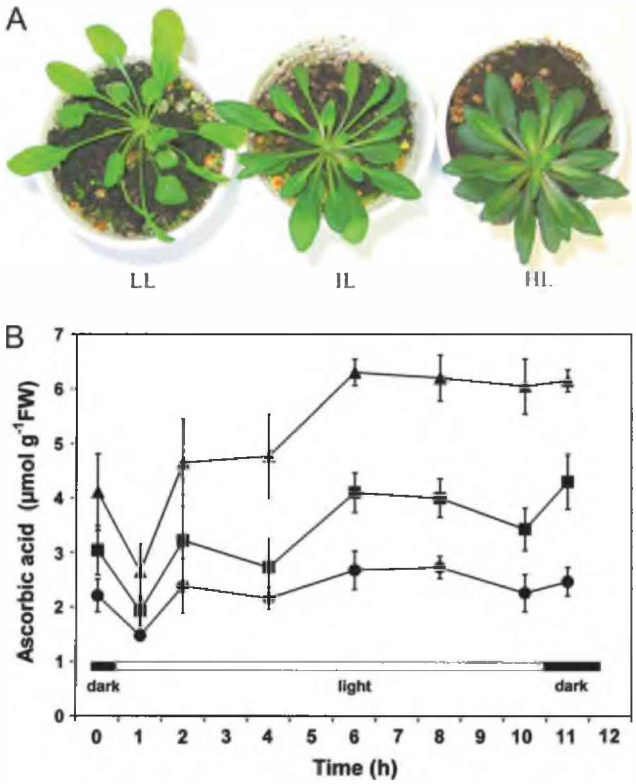

Fig. 1. Effect of growth light on *Arabidopsis* rosette phenotype (A) and leaf AA contents (B). (A) Plants were grown under LL (left; 50 μmol photons m^{-2} s^{-1}). IL (middle; 100 μmol photons m^{-2} s^{-1}). or HL (right; 250 μmol photons m^{-2} s^{-1}). (B) Plants were grown for 2 weeks under LL (circles; 50 μmol photons m^{-2} s^{-1}) and then transferred for a further 2 weeks to either LL, IL (squares; 100 μmol photons m^{-2} s^{-1}), or HL (triangles; 250 μmol photons m^{-2} s^{-1}). The extent of the light and dark periods is indicated at the bottom of (B). Data represent the median ±SE of at least three independent experiments.

Leaf AA contents tended to decrease during the first hour of illumination, but then increased once more, reaching values determined by the light level in which the leaves were grown. Leaf AA contents were highest in HL leaves and lowest in LL leaves (Fig. 1B). Leaf DHA contents were <10% of the total pool in all growth conditions. While interpretation of the DHA data must be regarded with caution as all values fall within the standard error of the AA measurements (Fig. 1B), there was a trend towards increased leaf DHA contents during the first hour of illumination, with values increasing to 1.9, 4.5, and 6.4%, respectively, for the LL, IL, and HL plants.

The effect of irradiance on the capacity of leaves to synthesize/accumulate AA

The capacity to synthesize AA *in vivo* was evaluated by feeding detached leaves with 30 mM L-GalL overnight (Table 1A). Under these conditions, leaves from HL plants accumulated twice as much AA as leaves from LL plants, while the leaves of IL plants had >50% more AA than the LL leaves (Table 1A). At HL, maximal

extractable L-GalLDH activity was twice that measured in LL leaves under the same conditions. Similarly, IL leaves had 60% higher L-GalLDH activities than LL leaves (Table 1A). However, the abundance of L-GalLDH mRNA was similar for all light treatments (data not shown).

The effect of irradiance on leaf respiration and the capacity to regenerate AA

Whole leaf respiration rates were lowest in the LL leaves and highest in the leaves of plants grown at HL (Table 1B). The capacities of both the CCO and AOX electron transport pathways were increased by increasing growth light intensities (Table 1B). Growth at HL increased maximal extractable leaf DHAR and MDHAR activities compared with IL- and LL-grown leaves (Table 1C). However, the irradiance-dependent increase in MDHAR activity was only significant in the HL leaves relative to the LL-grown leaves (Table 1C).

The respiration rate in isolated leaf mitochondria was measured using different substrates (Table 2). While values for NADH- and succinate-dependent respiration were similar under all light regimes, malate–pyruvate-dependent respiration increased in IL and HL plants compared with those grown at LL. In agreement with this observation, the leaves of plants grown at HL had three times as much cytochrome *c* and CCO activity as those grown at LL (Table 2). In IL, values for cytochrome *c* and CCO activity were double those of the LL treatment (Table 2).

Production of transgenic Arabidopsis *lines with altered levels of AOX protein and respiratory capacities*

Transgenic *A. thaliana* plants expressing a cDNA encoding AOX1a (At3g22370) in the sense and antisense orientations were used to elucidate the role of AOX in the synthesis and accumulation of AA. Twelve homologous sense lines which showed AOX accumulation were isolated together with 19 antisense lines that had decreased AOX levels (data not shown). Western blot analysis with specific antibodies to AOX revealed that the overexpressing lines such as S5 constitutively accumulated much larger amounts of AOX protein (Fig. 2A). The enhanced content of the AOX protein in the overexpressing lines was readily detected in comparisons with total leaf protein extracts from wild-type leaves (Fig. 2A). Antimycin induced increases in the detectable amounts of AOX protein in the mitochondria of wild-type leaves (Fig. 2B). In contrast, the

Table 1. *Effect of irradiance on the capacity for leaf AA synthesis and accumulation (A), respiration (B), and MDHAR and DHAR activities (C)*

Plants were grown at low (50 μmol photons m^{-2} s^{-1}). intermediate (100 μmol photons m^{-2} s^{-1}). and high (250 μmol photons m^{-2} s^{-1}) irradiances. The capacity for AA accumulation was measured in detached leaves incubated overnight in 30 mM L-GalL (10 h dark followed by 6 h light). Leaf AA contents were determined after 6 h light. Values are the means±SE of 3–5 independent experiments. Data with the same letters represent a statistically homogenous group (ANOVA, $P \leq 0.05$).

	Low	Intermediate	High
(A) The capacity for AA synthesis			
AA accumulation (μmol g^{-1} FW)	6.2±0.8 a	10.7±0.4 b	13.5±0.7 c
L-GalLDH activity (nmol cytochrome *c* mg^{-1} protein min^{-1})	1.7±0.36 a	2.7±0.25 b	3.5±0.50 b
L-GalLDH content (AU mg^{-1} protein)	4.3±0.54 a	8.1±0.80 b	8.5±0.87 b
(B) Leaf respiration [O_2 uptake (μl O_2 g^{-1} FW min^{-1})]			
Total respiration	4.7±0.4 a	7.1±0.2 b	8.2±0.9 b
KCN-resistant	1.7±0.1 a	2.9±0.7 ab	3.4±0.3 b
SHAM-resistant	3.5±1.0 a	5.7±1.0 ab	7.3±0.9 b
(C) DHAR and MDHAR activities [enzyme activity (nmol mg^{-1}protein min^{-1})]			
DHAR	377.0±52 a	516.0±40 b	720.0±28 c
MDHAR	27.8±4 a	33.4±6 a	47.7±2 b

Table 2. *The effect of growth irradiance on the respiratory rates measured in isolated leaf mitochondria and on the cytochrome* c *content and CCO activities of leaf homogenates*

Values are the means±SE of three independent experiments. Data with the same letters represent a statistically homogenous group (ANOVA, $P \leq 0.05$).

Irradiance	Respiratory activity (nmol O_2 mg $protein^{-1}$ min^{-1})			Cytochrome *c* content (AU U^{-1} protein)	CCO activity (μmol cytochrome *c* mg^{-1} protein min^{-1})
	NADH	Malate–pyruvate	Succinate		
Low	186±15	55±13 a	109±18	6.7±3 a	39±11 a
Intermediate	148±27	70±18 ab	141±24	13.1±1.4 a	55±9 a
High	187±30	97±3 b	144±25	21.2±1.7 b	100±2 b

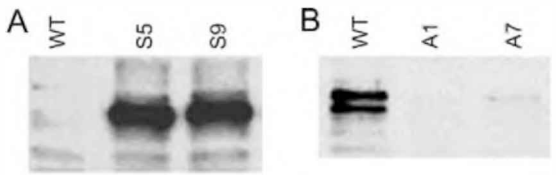

Fig. 2. The relative amounts of AOX protein in wild-type (WT) and transgenic lines expressing the transgene in the sense or antisense orientations. A comparison of the abundance of the 32 kDa AOX protein detected by specific antibodies in leaf extracts from S5 and S9 sense and WT plants (A) and in mitochondria isolated from WT and A1 and A7 antisense lines treated with antimycin A (B). For (A), leaf samples (0.1 g) were harvested from 3-week-old plants and ground in 800 µl of sample loading buffer. After centrifugation at 13 000 rpm. for 1 min, the soluble proteins were heated in a boiling water bath for 2 min. Samples (10 µl) were loaded in individual lanes on the SDS gels. For (B), leaves (0.5 g per sample) were detached from 4-week-old plants and vacuum infiltrated for 5 min with 10% strength MS medium containing 10 µM antimycin A. Intact mitochondria were isolated 16 h after infiltration. Samples (10 µg of mitochondrial protein) were loaded on the SDS gels.

antisense lines such as line A1 did not accumulate AOX even after treatment with antimycin A (Fig. 2B). On the basis of such western blot analyses, two lines (S5 and S9) that showed constitutively enhanced AOX protein abundance (Fig. 2A) and two lines (A1 and A7) that showed very low AOX protein abundance even after antimycin A treatment (Fig. 2B) were selected for further study.

The synthesis and accumulation of AA in AOX transformants

Leaves from antisense lines A1 and A7 had similar levels of AA to the wild-type at both LL and HL (Table 3). In contrast, the overexpressor lines S5 and S9 tended to have higher leaf AA contents than the wild-type and the antisense plants (Table 3). This effect was most marked in line S5, but was also observed in S9 particularly in leaves exposed to HL (Table 3). Similarly, when leaves were fed with AA precursor, L-GalL, the S5 line showed a significant increase in AA (production or maximal synthesis capacity) compared with the wild-type and antisense lines (Table 3). Mitochondria isolated from the S5 line showed a significant increase in the capacity to synthesize AA compared with the WT and A1 lines (Table 3). The level of DHA was similar in all lines and not greatly affected by modulation of AOX (data not shown).

To determine whether the increase in AA production observed in the AOX-overexpressing lines is linked to enhanced L-GalLDH activity or cytochrome *c* availability, these parameters were measured in mitochondria isolated from the different lines (Table 4). All genotypes had similar levels of L-GalLDH activity whether leaves were measured at LL (Table 4), or after 4 h exposure to HL (data not shown). However, both antisense lines have significantly more cytochrome *c* than the wild type or the S5 and S9 lines (Table 4, first column).

Discussion

While the control of AA biosynthesis by light and by respiration has been reported previously (Bartoli *et al.*, 2000, 2005; Smirnoff, 2000; Millar *et al.*, 2003), very little attention has been paid to integration of these key controls. In the present work, relationships between light and respiration in the control of AA synthesis have therefore been explored, with particular focus on the role of the AOX pathway. Although the relative engagement of the CCO and AOX pathways as a result of the treatments applied here has not been studied, it is shown that light enhances the capacities for these pathways together with an increased capacity for AA synthesis and accumulation.

The COX pathway includes complexes III and IV and is strongly regulated by the proton gradient between the matrix and the intermembrane space. In contrast, the AOX pathway shunts electrons away from complexes III and IV and prevents over-reduction of component electron acceptors in situations of excessive NADH availability (Vanlerberghe and McIntosh, 1997). Hence, it has been proposed that the role of this electron-dissipating pathway may be to minimize ROS formation by over-reduced electron transport chain components, particularly in the ubiquinone pool (Vanlerberghe and McIntosh, 1997).

Methyl jasmonate and jasmonic acid are important triggers mediating many plant stress responses including AA synthesis and accumulation (Wolucka *et al.*, 2005). Like AA synthesis, AOX expression is stimulated by biotic or abiotic stresses (Vanlerberghe and McIntosh, 1992; Gonzales-Meler *et al.*, 2001; Ordog *et al.*, 2002). Similarly, increased engagement of the AOX pathway has been demonstrated following exposure to stress (Ribas-Carbo *et al.*, 2000, 2005*b*). It should be noted that increased AOX expression and protein content are not necessarily indicators of increased engagement of the enzyme (Guy and Vanlerberghe, 2005). To date, the only available technique by which the partitioning of electrons between the two pathways can be measured *in vivo* is the oxygen isotope fractionation technique (Robinson *et al.*, 1992; Day *et al.*, 1996; Ribas-Carbo *et al.*, 2005*a*).

There have been no studies to date on the relationship between the capacity for AA synthesis in the mitochondria and the engagement of the AOX pathway. However, AOX activity is regulated by modulation of the redox state of the enzyme protein. This suggests that like L-GalLDH and AA synthesis (Bartoli *et al.*, 2005), AOX activity can be regulated by cellular redox perturbations. AOX is present in plant mitochondria as a dimeric di-iron homodimeric protein, with the two subunits linked by a disulphide bridge (Anderson and Nordlund, 1999). The reduced form is more active than the covalently linked oxidized form, but the regulation of the enzyme *in vivo* is far from understood (Millenaar *et al.*, 2001, 2002). For example, it was found that the lack of a detectable correlation between AOX

Table 3. *A comparison of AA synthesis and accumulation in wild-type* Arabidopsis *and in transformed lines either overexpressing (S5 and S9) or underexpressing (A1 and A7 AOX)*

Plants were grown at LL (50 μmol photons m^{-2} s^{-1}) and measurements were made either at this light level or after 4 h at high light (250 μmol photons m^{-2} s^{-1}). AA production was measured in detached leaves incubated in 30 mM L-GalL. Values are the means±SE of 3–4 independent experiments. Groups with the same letter in the same column are not statistically different from each other (ANOVA, $P \leq 0.05$).

Transformed lines	Whole leaf			Isolated mitochondria
	AA content (μmol g^{-1} FW)		AA production (μmol g^{-1} FW)	AA production (nmol mg^{-1}protein h^{-1})
	Low light	High light	High light	Low light
A1	2.90±0.5 abc	3.86±0.50 bcd	2.60±0.04 a	0.84±0.04 a
A7	2.66±0.4 ab	4.05±0.38 cd	3.19±0.35 ab	–
WT	2.36±0.4 a	3.80±0.23 bcd	2.85±0.25 a	0.96±0.04 a
S9	2.95±0.1 abc	4.61±0.7 de	3.58±0.15 b	–
S5	3.88±0.2 bcd	5.38±0.23 e	4.95±0.26 c	1.28±0.04 b

Table 4. *Cytochrome* c *contents and* L*-GalLDH activities in leaves and in mitochondria isolated from WT and AOX transgenic lines grown at LL (50 μmol photons* m^{-2} s^{-1}*)*

Values are the means±SE of 3–4 independent experiments. Groups with the same letter in the same column are not statistically different from each other (ANOVA, $P \leq 0.05$). A1 is significantly different from the rest of the treatments at $P \leq 0.1$).

Transformed lines	Whole leaf (U g^{-1} FW)		Isolated mitochondria (U mg^{-1} protein)	
	Cytochrome *c*	L-GalLDH	Cytochrome *c*	L-GalLDH
A1	40.2±7 ab	16.5±5	5.2±0.34	1.0±0.1
A7	46.0±5 b	9.0±3	nd[a]	nd
WT	26.0±6 a	16.0±4	7.1±1.2	1.5±0.3
S5	26.4±3 a	17.2±4	6.5±1.2	1.5±0.2
S9	24.3±10 a	17.0±3	nd	nd

[a] nd, not determined.

capacity and activity in the complex I-deficient CMSII mutant is not caused by a marked difference in the redox state of the enzyme protein, but it is related to isoform-specific changes in the expression of *AOX* genes (G Vidal, M Ribas-Carbo, M Garnier, G Dubertret, A Rasmusson, C Foyer, R De Paepe, unpublished data).

Evidence is provided here of the co-ordination of different components of the ROS-producing and defence systems within the plant mitochondrial electron transport chain. It is shown that the capacity for AA synthesis is increased together with respiratory capacity at high light. Moreover, it is shown that AA synthesis and accumulation is affected when the capacity of the AOX enzyme is manipulated in transgenic plants. These results allow the following conclusions.

Light is a major driver of AA accumulation in the light and disappearance in the dark

The results presented here emphasize the major effect of growth irradiance on leaf AA accumulation as previously described (Smirnoff, 2000). The results obtained with *Arabidopsis* leaves are almost identical to those reported previously for barley (Smirnoff, 2000). Light increases the capacity of *Arabidopsis* leaves to produce AA and increases L-GalLDH activities. These results agree with those obtained in previous studies showing that growth light enhances leaf AA synthesis and accumulation (Imai *et al.*, 1999; Gatzek *et al.*, 2002). Similarly, spinach leaves exposed to a very low light (20–25 μmol photons m^{-2} s^{-1}) show higher AA contents and capacities for AA synthesis than leaves stored in the dark (Toledo *et al.*, 2003). The stimulatory effect of light on AA production and accumulation in the diurnal cycle was not due to effects on L-GalLDH transcription, since light had no detectable effects on L-GalLDH transcript or to day/night variations in protein abundance (data not shown). Light therefore increases the capacity of *Arabidopsis* leaves to produce AA through other mechanisms that serve to increase L-GalLDH activity, such as redox regulation as discussed previously (Bartoli *et al.*, 2005).

It is shown here that the capacity to regenerate AA from its oxidized forms is enhanced in the HL-grown leaves, suggesting that amelioration of the recycling systems backs up the enhanced capacity for synthesis to ensure that the cellular ascorbate pool remains in the reduced state. Recycling of AA from its oxidized forms is an important process maintaining the AA pool (De Gara and Tommasi, 1999). Transgenic plants overexpressing DHAR were able to maintain a higher level of leaf AA accumulation (Chen *et al.*, 2003). Similarly, wheat cultivars with constitutively higher AA contents have higher recycling enzyme activities than cultivars with low leaf AA levels (Bartoli *et al.*, 2005). The importance of DHAR activity under HL conditions is documented in tropical fig leaves lacking DHAR activity, whose leaves turn yellow and have high flavonoid contents under HL (Yamasaki *et al.*, 1999).

AA turnover in plant tissues is considered to be high, having values of ~2% h^{-1} (Imai *et al.*, 1999; Pallanca and Smirnoff, 2000). The data presented here show that light increases the AA pool size and hence turnover. This is in agreement with data for peas where tissue AA was manipulated by feeding (Pallanca and Smirnoff, 2000).

These authors showed that the AA breakdown rate is directly proportional to pool size, irrespective of light. At least one pathway of AA breakdown is located in the apoplast (Green and Fry, 2005). Leaves that have higher AA levels transport more AA to the apoplast, whereas leaves of *vtc* mutants that have much reduced leaf AA contents have no apoplastic AA (Veljovic-Jovanovic *et al.*, 2001). These results illustrate the complexities of AA homeostasis in leaves where synthesis, transport, and degradation in different cellular compartments contribute to the control of overall levels of leaf AA accumulation.

Both of the mitochondrial electron transport pathways are important for the synthesis of AA

The oxidation of L-GalL depends on the content of L-GalLDH, and on mitochondrial electron transport chain activity. Light not only enhances L-GalLDH activity but it also increases respiration and the amount of cytochrome *c* to support higher CCO activities and enhanced AA synthesis. The observation that L-GalLDH activities and the amount of L-GalLDH protein only increased in IL plants compared with those grown at LL, with no further increases observed under HL conditions, suggests that these parameters have only a limited capacity for adjustment to available light. Moreover, it has previously been shown that leaf AA content is not determined by the amount of L-GalLDH present in the tissue (Bartoli *et al.*, 2005). Hence the enhanced capacity for respiration that leads to higher cytochrome *c* levels may also have a beneficial effect on AA synthesis.

A higher AOX capacity enhances AA synthesis

The physiological and regulatory association between the L-GalLDH protein and the respiratory electron transport chain might also have importance in adjusting leaf AA accumulation to light levels (Bartoli *et al.*, 2000; Millar *et al.*, 2003). Rotenone and KCN (but not antimycin A) block AA synthesis by isolated mitochondria, suggesting that the availability of oxidized cytochrome *c* and the activity of the mitochondrial complex I influence the oxidation of L-GalL. The capacities of both the cytochrome *c* and AOX pathways increase with irradiance. Changes in flux through these pathways may contribute to AA formation in different ways. Higher throughput in the cytochrome *c* pathway would require a bigger pool of electron acceptors for L-GalL oxidation, while enhanced capacity of the AOX pathway would favour AA synthesis by maintaining the cytochrome *c* pool in a more oxidized state, especially under HL conditions, as it prevents over-reduction of mitochondrial transporters under these conditions (Millar and Day, 1997). The amount of AOX protein present in the mitochondria of the transformed *Arabidopsis* leaves affected both AA synthesis and accumulation. Overall, the rate of AA production in the leaves of the S9 and S5 sense lines was higher than that of either the wild-type or the antisense plants. While the relationship between AOX expression and AA production was not significant at all time points, there is a clear correlation at 4 h HL, a time point when the availability of L-GalL is not limiting (Table 3). This increase was not linked to either enhanced L-GalLDH protein or cytochrome *c* availability, suggesting that enhanced engagement of AOX occurs in these lines. While the abundance of the AOX protein is not necessarily linked to engagement of the AOX pathway (Guy and Vanlerberghe, 2005), the higher AOX capacity measured in similar over-expressing *Arabidopsis* lines was reported to decrease ROS production (Umbach *et al.*, 2005). Transformed *Arabidopsis* plants with altered AOX levels showed modified KCN-stimulated mitochondrial ROS production and related gene expression (Umbach *et al.*, 2005). Hence, a higher AOX capacity in these conditions favours ROS accumulation (Umbach *et al.*, 2005) and enhances the capacity for AA synthesis as demonstrated in this study. Presumably, the greater AOX capacity in the overexpressing lines increases the availability of oxidized cytochrome *c*, which is the electron acceptor in the last step in AA synthesis. Interestingly, the antisense lines have significantly more cytochrome *c* than the wild type or the S5 and S9 lines (Table 4, first column), suggesting a complex interaction between the CCO and AOX pathways that would also directly affect the regulation of AA production.

The increments in cytochrome *c* content were calculated here on the whole leaf protein basis. This might reflect an increasing number of mitochondria per cell, or enhanced respiration per mitochondrion, or both, under HL treatment (Lewis *et al.*, 2000; Noguchi *et al.*, 2005). Growth under LL diminishes the rate of respiration and number of mitochondria per cell, while HL increases respiration and photosynthesis simultaneously by increasing the numbers of mitochondria and chloroplasts, as well as the capacity of each organelle for respiration and photosynthesis, respectively (Lewis *et al.*, 2000; Noguchi *et al.*, 2005).

Concluding remarks

The results presented here suggest that the mitochondrial electron transport chain exerts co-ordinated control over redox pathways involving AA and AOX capacity. The stimulatory effect of light could be due at least in part to effects on respiration. Similarly, respiratory controls appear to be important in determining the overall ability of leaves to produce and accumulate AA. Respiratory capacity has a pronounced effect on both these parameters despite apparent limitations on L-GalL synthesis within leaf cells that prevent accumulation of pathway intermediates. While more experiments are required to elucidate the effects of respiratory controls on the capacity for AA synthesis

in planta, the present results draw us to the conclusion that regulation of AA synthesis at the level of L-GalLDH activity in the mitochondria is important in determining leaf AA accumulation. Thus regulation of mitochondrial electron transport capacity and components modulates the ability of leaves to produce and accumulate AA.

These data show that plants use two interfacing strategies involving mitochondria for protection against high light. The enhanced capacity for AA production together with higher respiration rates and higher AOX capacities probably affords greater protection against uncontrolled oxidation as light availability is increased. The increased AOX capacities of transgenic *Arabidopsis* plants overexpressing AOX have been shown to decrease the probability of ROS accumulation (Umbach *et al.*, 2005). Similarly, higher AA production rates in situations where AOX capacity is increased lead to improved AA availability for ROS detoxification. These results provide evidence for the hypothesis that mitochondrial electron transport pathways make a key contribution to the control of whole cell redox homeostasis and signalling (Foyer and Noctor, 2005).

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